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Making immortalized cell lines from embryonic mouse kidney, to use as an alternative to in vivo experiments in renal research.

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Summary:

Immortalized cell lines derived from embryonic mouse kidneys are useful tools for exploring signalling pathways, morphogenetic mechanisms and gene function in renal development: they also provide a means for efficient first-round screening of panels of small molecules intended to combat renal pathologies such as the development of cysts, and such cell line-based screening can allow a valuable reduction in the numbers of animals needed for a given line of research. This chapter presents a simple method for generating cell lines from the '*Immortomouse*', which carries a temperature-sensitive SV40 antigen, under the control of an interferon-regulated promoter.

Key words: kidney ureteric bud metanephric mesenchyme, SV40, immortomouse, 3Rs

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Running head: kidney cell immortalization

1. Introduction

Transgenic knockout mice, and the various techniques for renal organ culture explained elsewhere in this volume, provide powerful methods for manipulating gene function in the context of a whole developing kidney. They have been largely responsible for our current understanding of renal development (1-6). There are studies, though, for which whole-organ experiments are inappropriate. Biochemical measurement of the protein phosphorylation response to activation of a signalling pathway, for example, is easier when only one cell type is present. For studies like this, it is very helpful to have a cell line that represents the cell type of interest and that can be grown in flasks to very large numbers. Cell lines that represent particular components in the developing kidney also show great promise for high-throughout screens for the ability of small molecules to affect cell behaviour. This might be for basic research in identifying important signalling proteins, or for applied research in identifying drugs able to interfere with pathways that cause cystic disease, for example. Performing such screens in whole animals would both be very expensive, financially and ethically, and very slow compared to rapid screening in cell culture.

It is difficult to raise cell lines from the kidneys of wild-type mouse embryos, possibly because each cell type requires survival signals from another (7). This problem can be mitigated by immortalizing cells artificially. A straightforward approach to this, and one that is safer than using vectors capable of immortalizing any cell they transfect, is to begin with kidneys of the *Immortomouse* strain (8). This mouse expresses a temperature-sensitive version of the SV40 large T antigen, under the control of an interferon-inducible promoter. Both interferon and temperature can therefore be used to modulate SV40-based immortalizing activity. Cells isolated from such a mouse can be grown in culture at 33°C with gamma-interferon (IFN-γ), and sub-cloned into separate lines.

It is important to note that cell lines produced in this way, while showing many of the properties of their parent tissues, are not exactly the same as them because they are selected for genetic and epigenetic changes that facilitate growth in culture. Many of the cell lines we have made, for example, express correct sets of markers and show responses to signals in the manner of their cell type of origin, but they will not re-integrate properly into a disaggregation / reaggregation culture system (9). The limitation is also true for the widely used mouse kidney cell lines M15, mIMCD3 and MDCK. Nonetheless, such cell lines can be excellent models for morphogenesis and cell signalling.

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2. Materials

2.1 Hardware:

- CO_2 incubator capable of running at 33˚C, and one running at 37˚C (see note 1).
- Dissection microscope (we use a Zeiss Stemi 2000)

- Fine forceps
- Syringe needles (BD Microlace-3 25G 0.5x16mm) and 1.0ml disposable syringes for dissection (these needles are good dissecting instruments and are cheap enough to be discarded when blunt).
- Cloning cylinders, glass (C1059-1EA Sigma)
- Dow Corning® high-vacuum silicone grease (Z273554-1EA, Sigma)
- Cell culture dishes (Corning® 100mm TC-Treated Culture Dish ,Product #430167).
- T-25 tissue culture flasks (Corning. Cat. # 430639)
- 6-well cell culture plates, (Corning #3471)
- 24-well cell culture plates (Corning)

2.2 Animals

- H-2Kb tsA58 transgenic immortal mice (8).

2.3 Media

- Coating Solution: 2% solution of Matrigel (BD biosciences) in ice-cold 1:1 DMEM-F12 (Sigma D8437: see note 2). Make this freshly each day.
- Dissecting Medium : DMEM (Sigma D5546)
- Separation Medium: 2U/ml Dispase II in dissecting medium. This is remains active for about 2 weeks at 4˚C.
- Disaggregation Solution: 1x Trypsin EDTA (Invitrogen 25300-062)
- Immortalization Medium: 1:1 mix of DMEM-F12 (Sigma D8437) with 10% heat-inactivated fetal bovine serum (Invitrogen), IFN-γ 100u/ml (ProSpecBio cyt-358), 1% ITS supplement (Sigma I2521 – ie a final 1 in 100 dilution of the manufacturer's stock: this supplement contains 1 mg/ml insulin, 0.55 mg/ml human transferrin, and 0.5 μg/ml sodium selenite), glutamine, penicillin and streptomycin (these last three coming from a single stock solution; Invitrogen 10378016) and 1x antioxidants (Sigma A1345).
- Enriched Immortalization Medium: Immortalization Medium with a total of 200u/ml IFN-γ, 2% ITS and 2x antioxidants.
- ROCK inhibitor medium: Immortalization Medium with 10μM Y-27632 (Sigma Y0503).

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3. Methods

3.1 Initial culture of cells

1. Prepare culture dishes by placing 5ml of Coating Solution in each and leaving them on a flat surface at room temperature for a few hours. Rinse in dissecting medium before use.

2. Isolate metanephric rudiments from mouse embryos by microdissection in dissecting medium. It is assumed that anyone reading this chapter will be familiar with a suitable technique for doing this and it would take up too much space to explain it here. Detailed guidance is available in a previous volume of this journal (10).

2. (OPTIONAL – see Note 3) Isolate the individual renal tissue of interest. For our work on E11.5 kidneys, we separate metanephric mesenchyme from ureteric bud by incubating kidney rudiments for 5-10 minutes in room temperature Separation Medium, and then gently pulling the ureteric bud away from the mesencyhme using needles (see Fig 1); rinse in dissecting medium.

3. Disaggregate tissues to make a single-celled suspension. This is done by placing 3-4 E11.5 kidneys, or 8-10 isolated ureteric buds or 8-10 isolated mesenchymes, or about one E14.5 kidney crudely chopped into pieces, into Disaggregation Solution for 4-10 minutes at 37˚C. Using a glass pipette, transfer the tissue to an Eppendorf tube containing about 200µl Immortalization Medium. Leave it for 5-10 mins and then complete its dissociation by trituration (pipetting repeatedly into and out of a yellow Gilson tip). Monitor the cells on a microscope to verify that they are in single-celled suspension.

4. (OPTIONAL – sort cells by FACS or magnetic beads at this point – see Note 3).

5. Plate the cell suspension in coated cell culture dishes in 8mls ROCK inhibitor medium. Leave in a 33˚C incubator for 48h.

6. Replace the medium with Immortalization Medium (with no ROCK inhibitor). Incubate at 33˚C for 72h.

7. Repeat step 6.

7. Replace medium with Enriched Immortalization Medium and leave for 4-10 days, watching for the appearance of small proliferating clones of cells.

3.2 Cloning

8. When proliferating clones of 20-40 cells can be seen, use a marker pen to draw around them (on the underside of the dish – see note 4). Continue to incubate the dish until the clones each acquire about 150 cells.

9. Place a thin layer of silicone grease in a glass dish and autoclave it: this will result in a thin layer of sterile grease.

10. Press the bottom of the sterile cloning rings firmly on to the silicone grease film so that they acquire a thin coat of it.

12. Place Disaggregation Solution in an incubator to warm up to 37˚C.

11. Rinse the culture dish in sterile PBS, then place a cloning rings firmly over the clones in which you are interested (see note 5). Add 100µl warm Disaggregation Solution and leave at 37˚C for 2 mins.

12. Add 100µl Immortalization Medium to each cloning ring, recover the suspended cells and centrifuge at 2500rpm for 2 minutes. Discard the supernatant.

13. Resuspend the cells in 1ml Immortalization Medium and culture in a 24-well plate for 1-2 weeks at 33˚C, changing the medium every 3 days. Inspect the cells, and move to the next step when they reach about 85% confluence.

3.3 Expansion of clones

14. When the cells have reached about 85% confluence, rinse with 1x PBS, replace this with pre-warmed Disaggregation Medium for 2 minutes. Recover and centrifuge cells as in step 12 above. Re-plate in a 6-well plate in Immortalization Medium.

15. When 85% confluence is again achieved, passage the cells again, as in step 14, and transfer to a T25 culture flask. For reasons of economy, 50U/ml IFN- γ can be used.

16. When a culture has been established, it is worth testing whether it will grow without IFN- γ (adaptation to cell culture often means that the cells become IFN- γ independent. We detect expression of the SV40 transgene in our cell lines even without it – Fig 2).

17. Optional: test the expression of markers of interest by standard RT-PCR (Fig 2).

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4. Notes

1. The 33˚C should be in addition to a conventional one at 37˚C, because some of the enzyme digestions mentioned in this protocol require the higher temperature.

2. It is essential that coating medium be kept ice cold until coating begins (otherwise it will gel).

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3. It is possible to raise cell lines by culturing mixed cells from whole kidney rudiments, making clones and then identifying the cells each clone represents by a study of marker genes ('anchor genes' in the language of Thigarajan et al. (11), who list many). It may be much more efficient, however, to put effort into separating tissues to isolate only the desired cell type in the first place. When cell lines are being raised from young (<E12) kidneys, mechanical dissection may be all that is needed. Later, the structure of the organ becomes too intricate for this, so FACS or magnetic sorting is needed.

4. Choose only clones that are distant enough from other clones that a cloning ring can be put around them alone.

5. Choose no more than 8 clones in one dish: dealing with more introduces too much delay in which cells are being manipulated and reduces viability.

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Figures

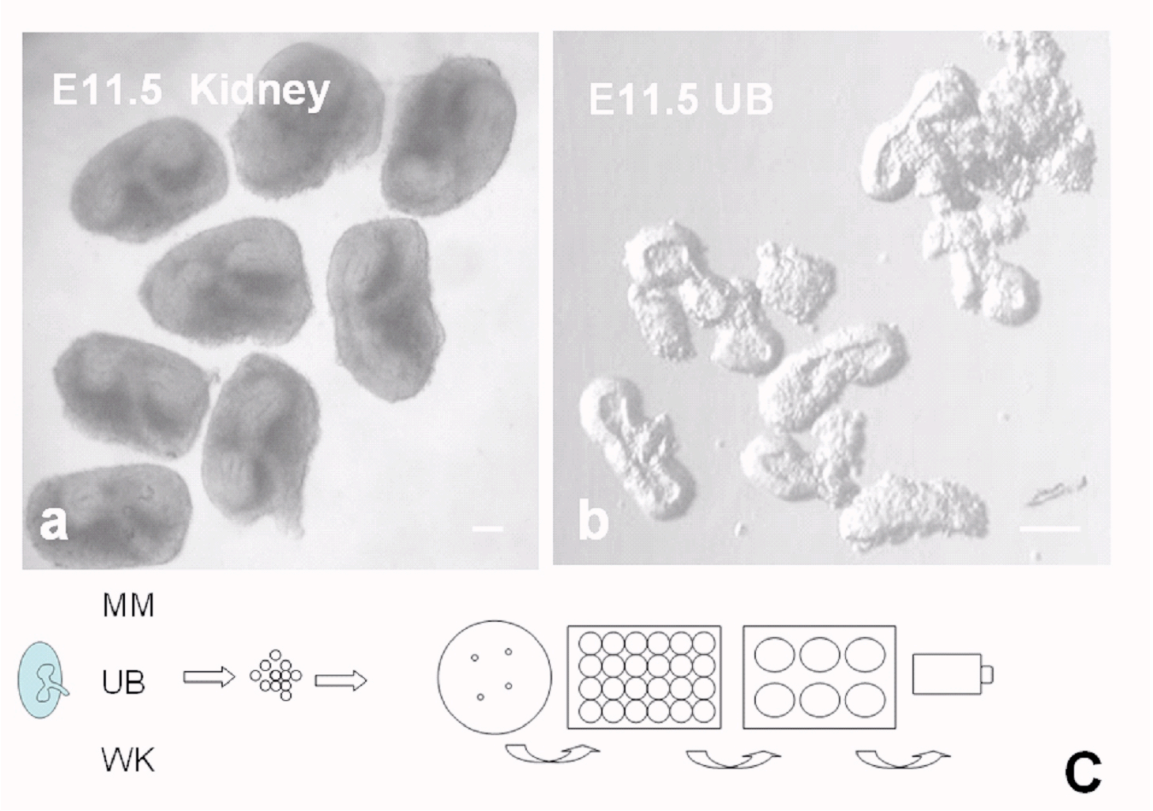

Fig1 **Micro-dissection of E11.5 kidney.** (a) At E11.5, the ureteric bud has made a 'T-shape' but no nephrons have formed. (b) Dispase digestion allows mesenchymes to be pulled away from utereric buds (the buds being shown herte, although there is some mesenchyme at the top right of the image). (c) A cartoon illustrating the sequence of events described in this chapter; MM= metanephrogenic mesenchyme, UB= ureteric bud, WK= whole kidney. Scale bar = 100μm.

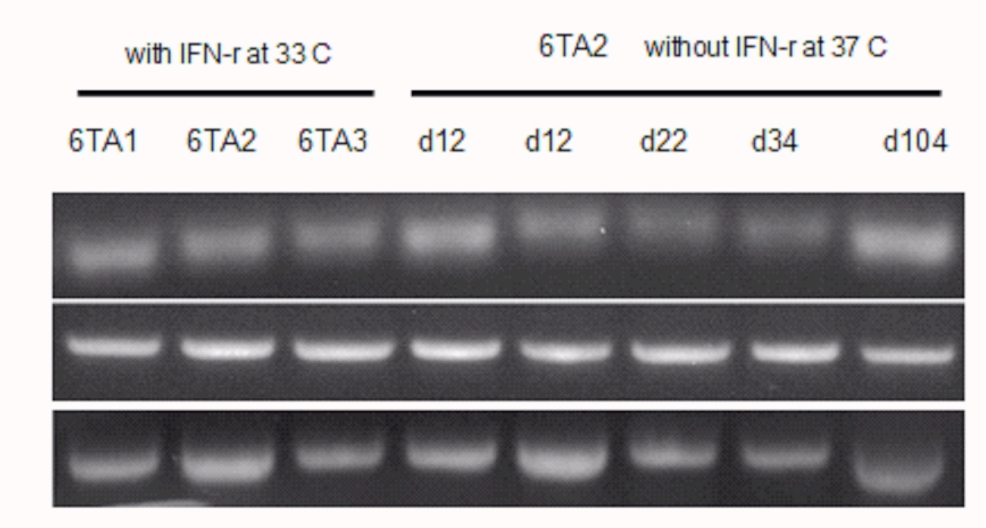

Fig . 2 Expression of SV40T in established lines with and without IFN-γ The figure shows RT-PCR detection of GAPDH (a control house-keeping gene), Wnt11 (a marker for ureteric bud tips, which we wished our these cell lines to represent) and the SV40 large T transgene. The SV40 large T was expressed even without addition of IFN-γ, and at both 33°C and 37°C, although coding for a temperature sensitive protein, it would not be expected to give rise to significant activity at 37°C. The numbers '6TA1' etc are clone numbers, and the d12, d22 etc were days of culture since subcloning.

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